# Tumor Necrosis Factor-α- and IL-4-Independent Development of Langerhans Cell-Like Dendritic Cells from M-CSF-Conditioned Precursors

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GM-CSF and transforming growth factor β (TGFβ) are required for the generation of Langerhans cells (LC), members of the dendritic cell (DC) family. Tumor necrosis factor α (TNFα) and IL-4 can enhance LC differentiation from human monocytes or $CD34^+$ progenitors. Here, we show that M-CSF-cultured DC precursors derived from $CD34^+$ progenitors resemble dermal $CD14^+$ cells and readily convert to LC-like DC in GM-CSF/TGFβ. The cells express Langerin, CD1a, and CCR6, migrate in response to CCR6 ligand CCL20, and contain Birbeck granules. TNFα and IL-4, added separately or together, have an inhibitory effect on LC differentiation. Cells differentiated in the presence of IL-4 and TNFα express low levels of CCR7. This suggests that M-CSF-conditioned DC precursors retain the capacity to efficiently undergo a differentiation program, giving rise to LC-like DC solely through the effect of GM-CSF and TGFβ.

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## INTRODUCTION

Langerhans cells (LC) represent a subset of dendritic cells (DC) found in the stratified epithelia of skin and mucosae (Maurer and Stingl, 2001). LC express CD1a but are distinguished from other DC by the presence of intracellular Birbeck granules, containing the C-type lectin Langerin (CD207) (Valladeau *et al.*, 2000) and the cell adhesion molecule E-cadherin, enabling tight interaction with epithelial cells (Tang *et al.*, 1993). Intra- or near-epithelial LC express CCR6 (Charbonnier *et al.*, 1999), the chemotactic receptor for the chemokine CCL20 produced by epithelial cells (Charbonnier *et al.*, 1999; Dieu-Nosjean *et al.*, 2000).

LC development requires transforming growth factor β (TGFβ) (Borkowski *et al.*, 1996), and human LC can be generated from $CD34^+$ hematopoietic progenitors cultured with GM-CSF, TGFβ, and tumor necrosis factor α (TNFα) (Caux *et al.*, 1992; Strunk *et al.*, 1996). Under these conditions, TNFα favors the outgrowth of $CD1a^+$ DC precursors (Caux *et al.*, 1992; Canque *et al.*, 1998), which express CCR6 but no CCR7 and migrate in response to CCR6 ligand CCL20 (Dieu *et al.*, 1998; Charbonnier *et al.*, 1999). In the presence of IL-4, LC differentiation is also enhanced by TNFα; however, the cells express CCR7 and migrate in response to CCR7 ligands CCL19 or CCL21 (Geissmann *et al.*, 2002; Arrighi *et al.*, 2003). The localization of cells with LC phenotype in lymph nodes of patients with dermatopathic lymphadenitis may be correlated with such TNFα/IL-4-dependent differentiation pathways (Geissmann *et al.*, 2002). It has been shown that human dermis contains LC precursors, which express CCR6 and CD14 and already contain Birbeck granules (Larregina *et al.*, 2001). Upon culture in GM-CSF/TGFβ, the cells lose CD14 and acquire CD1a and E-cadherin. This suggests that LC development can follow a TNFα/IL-4-independent differentiation program with homing affinity for the epithelium or a TNFα/IL-4-driven differentiation program with migration to draining lymph nodes.

We have previously observed that dermal $CD14^+$ cells express TNF-related activation-induced cytokine (TRANCE) and TRANCE-receptor (R) (Cremer *et al.*, 2002), members of the TNF family that are involved in cell viability (Wong *et al.*, 1997). Cells that share these and other antigenic markers with dermal $CD14^+$ cells can be obtained by culturing day-6 $CD34^+$ progenitor-derived $CD14^+$ cells in M-CSF (Cremer *et al.*, 2002). We will refer to these cells as dermal dendrocyte-like DC (DDL-DC) in order to distinguish them from day-6 $CD14^+$ cells. Recently, we have demonstrated that DDL-DC are permissive to dengue virus and HIV infection and can differentiate into immature DC when cultured in GM-CSF/IL-4 (Kwan *et al.*, 2005). Here, we investigate further their cell plasticity and address the question of whether DDL-DC can differentiate into LC.

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*Abbreviations: DC, dendritic cells; DDL-DC, dermal dendrocyte-like dendritic cells; LC, Langerhans cells; TRANCE, TNF-related activation-induced cytokine; TRANCE-R, TRANCE-receptor*

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## RESULTS

### CD34$^+$ hematopoietic progenitors differentiate into CD14$^+$ TRANCE$^+$ TRANCE-R$^+$ DDL-DC

We have previously shown that day-6 CD14$^+$ cells derived from CD34$^+$ progenitors can differentiate into CD14$^+$ factor XIIIa$^+$ TRANCE$^+$ TRANCE-R$^+$ cells in M-CSF-supplemented culture (Cremer *et al.*, 2002). We found that cells with similar phenotype could also be obtained by culturing the unseparated cell populations at day 6 in M-CSF (Figure 1a). The mean percentage of CD14$^+$ cells in the population was 72% ($\pm$1.7, $n=5$). The CD14$^+$ cells thus obtained were immunomagnetically purified and tested for expression of specific antigenic markers by flow cytometry. Figure 1b shows that the cells carried markers shared with the dermal CD14$^+$ subset, including factor XIIIa, DC-SIGN (CD209), but lacked CD1a and Langerin. We refer to these cells as DDL-DC.

### Differentiation of DDL-DC into CD1a$^+$ Langerin$^{Hi}$ cells

In order to assess the ability of DDL-DC to differentiate into LC, we cultured the cells in GM-CSF/TGFβ in the presence or absence of IL-4 or TNFα. We monitored LC differentiation by measuring intracellular expression of CD1a and Langerin. Figure 2a shows that DDL-DC cultured in GM-CSF/TGFβ acquired expression of CD1a and Langerin, IL-4, and TNFα′ however, inhibited differentiation into CD1a$^+$ Langerin$^{Hi}$ cells. Differentiation of DDL-DC into CD1a$^+$ Langerin$^{Hi}$ cells is accompanied by loss of CD14 and DC-SIGN (data not shown). The proportion of CD1a$^+$ Langerin$^{Hi}$ cells obtained in GM-CSF/TGFβ varied between donors (38.7$\pm$14.4%) but the inhibitory effects of IL-4 and TNFα were always observed (5.3$\pm$3.5 and 8.3$\pm$2% positive cells, respectively) (Figure 2b). We next assessed the kinetics of differentiation by measuring CD1a and Langerin expression at 24-hour intervals over 3 days (Figure 2c). A small population of CD1a$^+$ Langerin$^{Hi}$ cells appeared at 24 hours and then rapidly increased in the following 48 hours. The inhibitory effect of IL-4 was observed at all time points. Cultures allowed to proceed beyond 3 days did not markedly increase the proportion of CD1a$^+$ Langerin$^{Hi}$ cells in either the presence or absence of IL-4 (not shown).

### GM-CSF/TGFβ induces differentiation of CCL20-responsive CCR6$^+$ cells

LC precursors express CCR6, the receptor for the chemokine CCL20, produced by epithelial cells (Dieu *et al.*, 1998; Charbonnier *et al.*, 1999). We assessed CCR6 expression by flow cytometry and response to CCL20 by $Ca_2$ flux and cell migration. DDL-DC lacked notable CCR6 expression, but a 24-hour incubation in GM-CSF/TGFβ upregulated CCR6 (Figure 3a). Addition of IL-4 to GM-CSF/TGFβ prevented CCR6 upregulation. The $Ca_2$ flow induced by chemokine receptor ligation was measured in response to CCL20 and in comparison to CCL2, the ligand for CCR2 that is widely expressed on the myeloid lineage (Figure 3b). DDL-DC showed weak CCL20-induced signaling (28 nM of $Ca^{2+}$) but a robust $Ca_2$ flow in response to CCL2. After a 24-hour culture in GM-CSF/TGFβ, the cells increased the CCL20-induced signaling (56.4 nM of $Ca^{2+}$) but reduced the response to CCL2. The cells cultured in GM-CSF/TGFβ

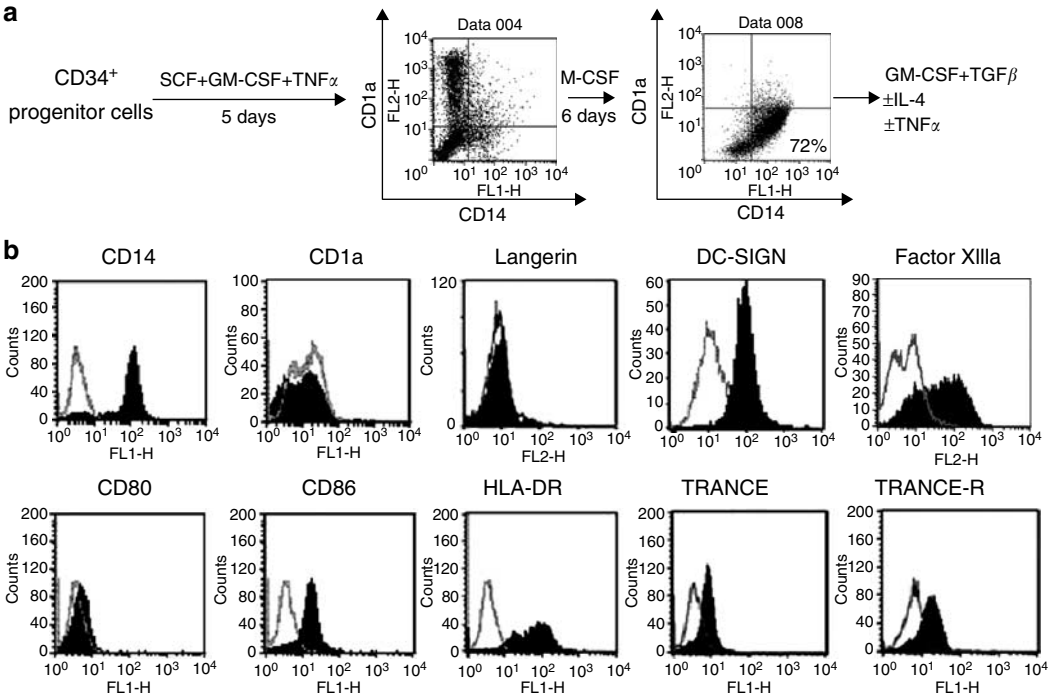


**Figure 1. Generation of DDL-DC from CD34$^+$ hematopoietic progenitors.** (**a**) Schematic overview of the culture conditions to obtain CD1a$^+$, CD14$^+$, double positive and negative cells at day 5 and a majority (72%) of CD14$^+$ cells at day 11. (**b**) Phenotype analysis of CD14$^+$-purified DDL-DC using the indicated antibodies. Expression of all markers was detected at the cell surface. Specific labeling is shown in black histograms and isotype controls in white histograms.

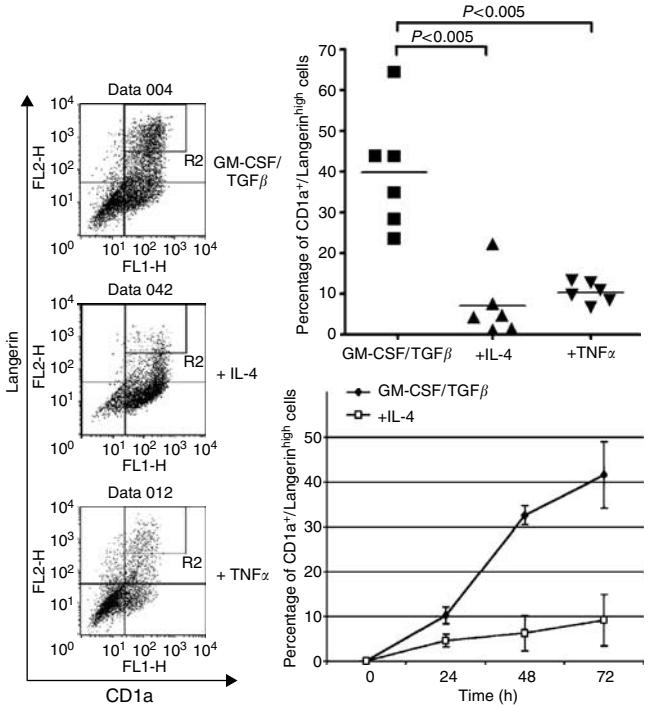


**Figure 2. DDL-DC differentiate into CD1a$^+$ Langerin$^{Hi}$ cells.** DDL-DC were cultured for 3 days in GM-CSF/TGFβ, alone or in the presence of IL-4 or TNFα. (**a**) Representative phenotype analysis of CD1a/Langerin expression. (**b**) The percentage of CD1a$^+$ Langerin$^{Hi}$ cells, electronically gated as shown in (**a**) and obtained in GM-CSF/TGFβ in the presence or absence of 1 ng/ml IL-4 or 1 ng/ml TNFα, is shown for each donor. Significance was evaluated using the *t*-tailed Student's test. (**c**) Kinetics of differentiation of DDL-DC into CD1a$^+$ Langerin$^{Hi}$ cells in GM-CSF/TGFβ in the presence or absence of IL-4. The percentage of CD1a$^+$ Langerin$^{Hi}$ cells was measured every 24 hours for 3 days and expressed as the mean ± SE of three experiments.

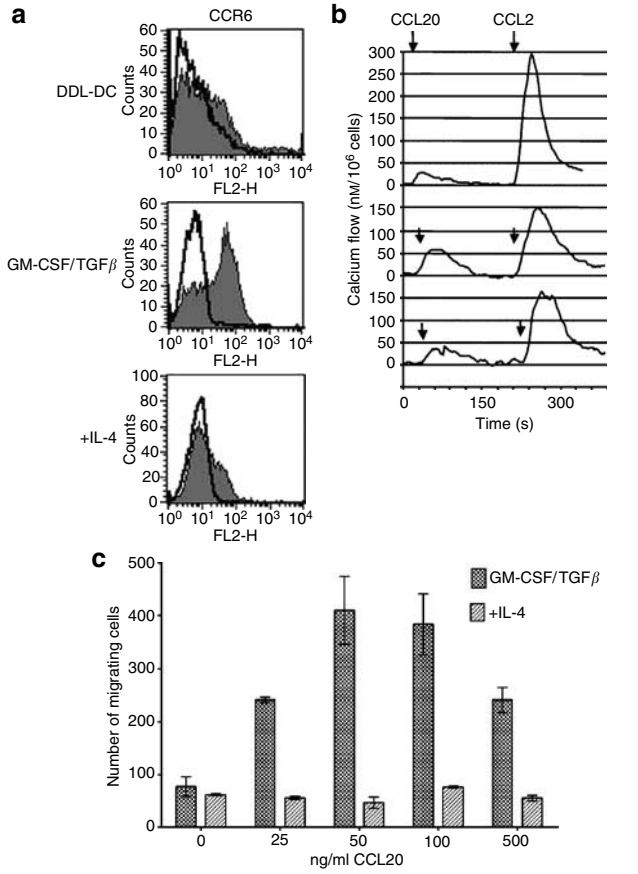


**Figure 3. GM-CSF/TGFβ induces differentiation of CCL20-responsive CCR6$^+$ cells.** (**a**) CCR6 expression of DDL-DC before and after a 24-hour culture in GM-CSF/TGFβ in the presence or absence of IL-4. Specific labeling is shown in gray histograms and isotype controls in white histograms. (**b**) Measurement of calcium flow in response to CCL20 or CCL2 ligation expressed as nM of mobilized calcium per $10^6$ cells. The arrow indicates the time of chemokine addition. The cells were as in (**a**). The experiment was performed twice using different donor CD34$^+$ cells with similar results. (**c**) GM-CSF/TGFβ- or GM-CSF/TGFβ + IL-4-cultured DDL-DC were tested for chemotactic migration in response to increasing concentrations of CCL20. The number of migrated cells is expressed as the mean ± SE of three independent experiments.

supplemented with IL-4 showed a CCL20 response similar to DDL-DC (32.8 nM of $Ca^{2+}$) but with reduced CCL2 signaling. Next, we tested the cells for migration in a CCL20 chemotactic gradient (Figure 3c). DDL-DC cultured in GM-CSF/TGFβ displayed prominent migration with a peak response at 50 ng/ml CCL20. Using a chemokine concentration range, a bell-shaped response curve was obtained characteristic of a chemotactic response. Cells that differentiated in the presence of IL-4 did not migrate at any of the CCL20 concentrations tested. Taken together, these data indicate that DDL-DC differentiate into cells with features characteristic of LC (CD1a, Langerin, CCR6 expression and CCL20 response) in response to GM-CSF/TGFβ. Addition of IL-4 or TNFα had inhibitory effects on LC differentiation.

### Effect of simultaneous presence of IL-4 and TNFα on LC differentiation

TNFα has been shown to improve LC differentiation from CD34$^+$ progenitors and monocytes in conditions where IL-4 is present (Geissmann *et al.*, 2002; Arrighi *et al.*, 2003). Therefore, we explored how the simultaneous presence of IL-4 and TNFα affected the formation of LC-like DC. DDL-DC were incubated for 3 days in GM-CSF/TGFβ in the presence or absence of TNFα or IL-4, separately or together. Figure 4a shows flow cytometry profiles of CD1a/Langerin expression in three experiments, Figure 4b summarizes the mean percentage of CD1a$^+$ Langerin$^{Hi}$ cells (gated in panel a), and Figure 4c the mean fluorescence index of Langerin expression (upper right quadrant in panel a). Addition of either TNFα or IL-4 to GM-CSF/TGFβ antagonized the formation of CD1a$^+$ Langerin$^{Hi}$ cells. TNFα had potent inhibitory effects at doses as low as 500 pg/ml. The simultaneous presence of TNFα and IL-4 led to a partial TNFα dose-dependent restoration of CD1a$^+$ Langerin$^{Hi}$ cells, but the percentage of CD1a$^+$ Langerin$^{Hi}$ cells was significantly smaller, and the expression level of Langerin remained significantly lower compared to the GM-CSF/TGFβ condition.

We next investigated expression of chemokine receptors CCR6 and CCR7 as well as maturation markers CD86, CD83,

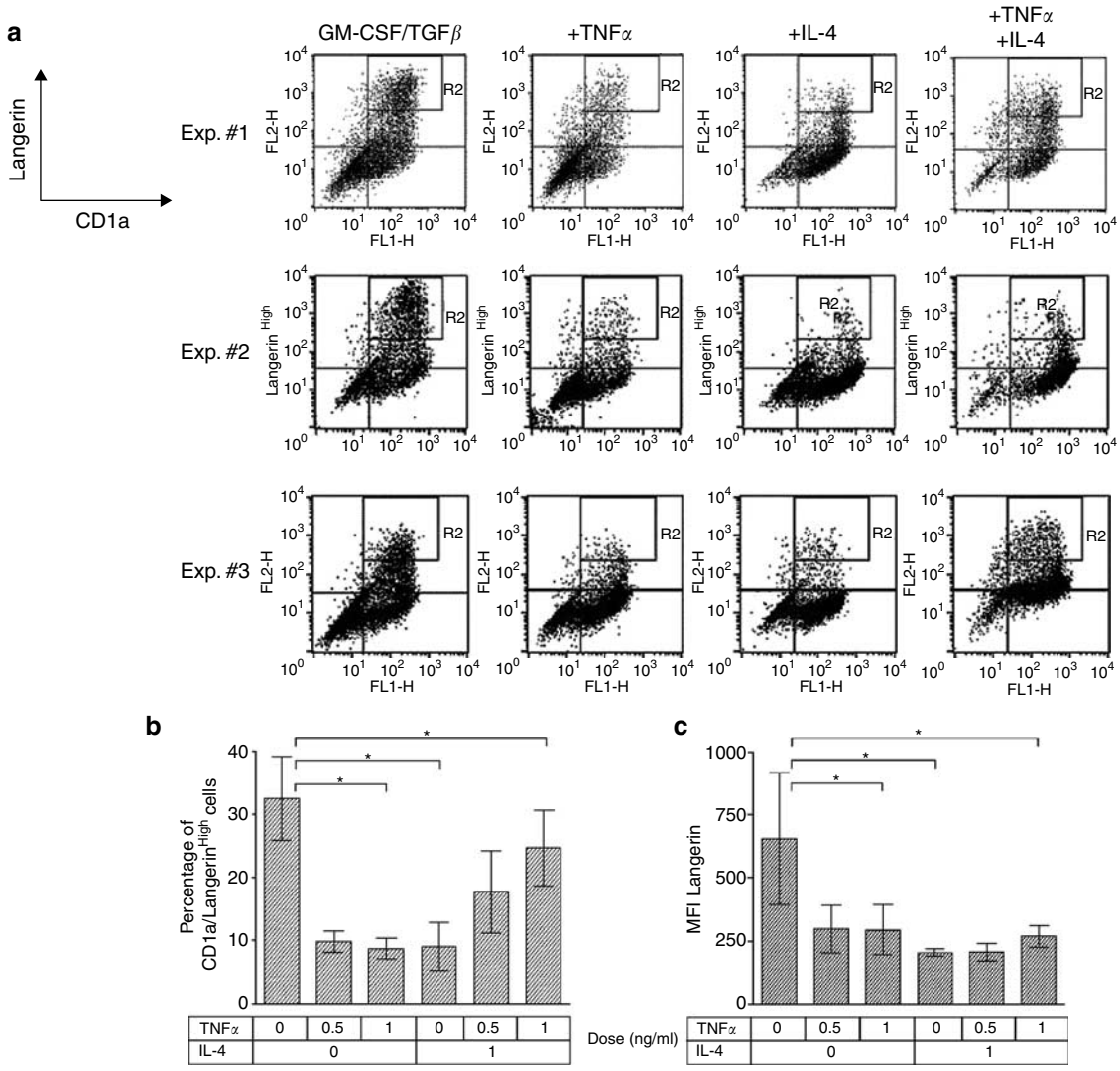


**Figure 4. TNFα and IL-4 inhibit LC differentiation, but simultaneous addition of both cytokines diminishes their inhibitory effect.** (**a**) DDL-DC cultured for 3 days in GM-CSF/TGFβ in the presence or absence of 0.5 ng/ml TNFα or 1 ng/ml IL-4, or in the presence of both TNFα and IL-4, were analyzed for CD1a/Langerin expression. The results of three experiments are shown. The CD1a$^+$ Langerin$^{Hi}$ population is gated. (**b**) Representation of the mean percentage ± SE of the CD1a$^+$ Langerin$^{Hi}$ cells for the three experiments. The CD1a$^+$ Langerin$^{Hi}$ cells were defined as located within the gate of (**a**). The doses (ng/ml) of TNFα and IL-4 are indicated below the histograms. Significance was calculated using the *t*-tailed Student's test (*$P<0.05$). (**c**) Representation of the geometric mean ± SE intensity index of fluorescence of Langerin expression in the upper right quadrant of (**a**). The doses of TNFα and IL-4 are indicated below the histograms. Significance was calculated using the *t*-tailed Student's test (*$P<0.05$).

CD80, and HLA-DR. As shown in Figure 5a, the cells generated in GM-CSF/TGFβ expressed cell-surface Langerin and CCR6 but virtually no CCR7. Addition of IL-4 induced low levels of CCR6 and CCR7. In the presence of TNFα, Langerin$^+$ cells expressed CCR6 but lacked CCR7. The simultaneous presence of IL-4 and TNFα resulted in a few Langerin$^+$ cells that showed similar expression of CCR6 and CCR7 as IL-4-treated cultures. The cells cultured in the different cytokine combinations lacked CD86, CD83, and CD80 expression. With respect to HLA-DR, TNFα-stimulated cultures comprised a small cell population expressing high levels of HLA-DR. We assessed whether the cells could respond to the dual maturation stimulus lipopolysaccharide (LPS)/anti-CD40. As shown in Figure 5b, the cells obtained under all conditions formed cell clusters, upregulated CCR7, HLA-DR, CD86, CD83, and CD80 concomitant with a decrease in cell-surface Langerin.

**GM-CSF/TGFβ-generated LC-like cells contain Birbeck granules**

We performed transmission electron microscopy on ultrathin-sectioned cells generated from DDL-LC in the presence of GM-CSF and TGFβ (Figure 6). Cells displayed dendritic morphology with slightly off-centered lobulated nuclei. The cytoplasm was devoid of multivesicular organelles typical of mononuclear macrophages, but contained Birbeck granules, cytoplasmic organelles forming a rod-shaped structure with double membrane junctions (Figure 6, inset) (Caux *et al.*, 1992). More open-ended tennis-racket-shaped granules were not seen. About 10% of the cells showed these rod-shaped Birbeck granules.

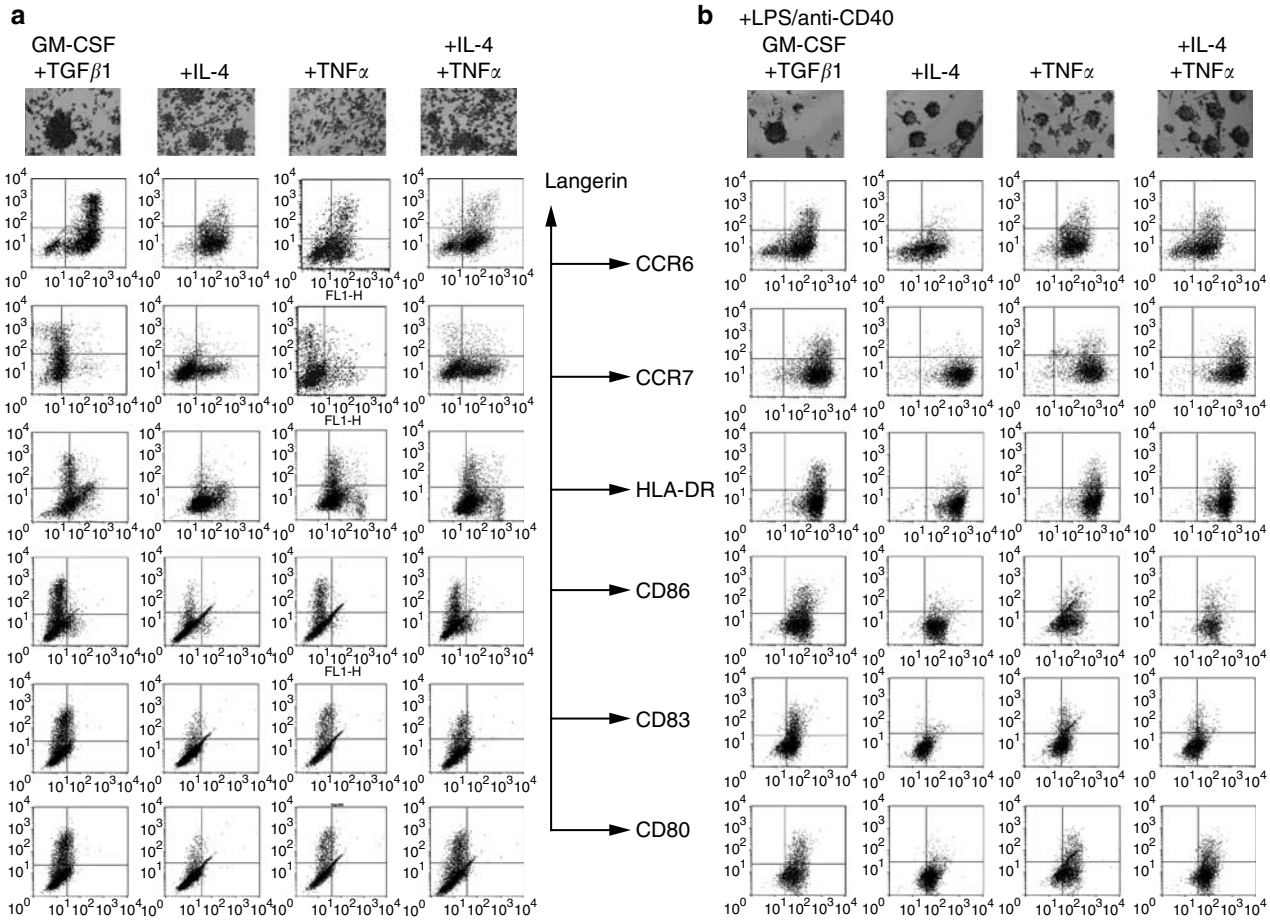


**Figure 5. GM-CSF/TGFβ-cultured cells mature in response to LPS/anti-CD40.** (**a**) DDL-DC cultured for 3 days in GM-CSF/TGFβ in the presence or absence of TNFα or IL-4, or in the presence of both TNFα and IL-4, were visualized by light microscopy and analyzed for expression of cell-surface Langerin versus CCR6, CCR7, HLA-DR, CD86, CD83, and CD80. The FACS plots for CCR6, CCR7, and CD83 are representative of five experiments and, for CD86, CD80, and HLA-DR, are representative of two experiments. (**b**) The same cells, as shown in (**a**), were stimulated for 24 hours with LPS/anti-CD40, visualized by light microscopy, and analyzed for cell-surface marker expression. For all flow cytometry analyses, expression levels were obtained after gating on total live cells, identified by forward and side scatter profiles.

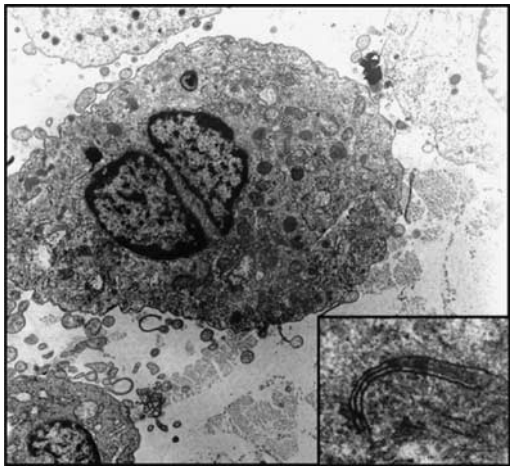

**Figure 6. Transmission electron microscopy visualizes Birbeck granules.** DDL-DC were cultured in GM-CSF/TGFβ for 6 days and processed for transmission electron microscopy. About 10/100 cells analyzed at a magnification of ×30,000 showed rod-shaped Birbeck granules (inset, lower left).

## DISCUSSION

In this report, we have shown that DDL-DC, which share phenotypic markers with the dermal $CD14^+$ cells, readily differentiate into LC-like DC upon culture in GM-CSF/TGFβ. The presence of either TNFα or IL-4 led to inhibition of LC differentiation, and simultaneous addition of IL-4 and TNFα could only partially restore LC generation.

The precursor cells were generated from $CD34^+$ hematopoietic progenitors in the presence of M-CSF, which induced loss of CD1a and acquisition of CD14. The cells, which we refer to as DDL-DC, expressed other markers characteristic of dermal $CD14^+$ mononuclear phagocytes including DC-SIGN (Turville *et al.*, 2002), factor XIIIa (Cerio *et al.*, 1989), TRANCE, and TRANCE-R (Cremer *et al.*, 2002, Kwan *et al.*, 2005). The data presented here show that, under the influence of GM-CSF/TGFβ, DDL-DC acquire features typical of LC: (1) high expression of Langerin, CD1a, and CCR6; (2) signal and migratory response to CCR6 ligand CCL20; and (3) Birbeck granules. This supports the idea that TGFβ and GM-CSF can be sufficient for LC formation from

dermal CD14$^+$ precursor cells (Larregina *et al.*, 2001). GM-CSF is required for LC formation from DDL-DC and cannot be omitted or replaced by M-CSF (data not shown) and DDL-DC differ from other progenitors in this manner (Larregina *et al.*, 2001; Mollah *et al.*, 2003). We have also tested for the requirement of fetal calf serum and found that differentiation of LC-like cells did not occur in serum-free culture medium (data not shown). The percentage of CD1a$^+$ cells that express high levels of Langerin was $38.7 \pm 14.4\%$, which compares favorably with other means of generating Langerin$^+$ cells from CD34$^+$ progenitors, reported as 25% using GM-CSF/TGFβ/IL-4/TNFα (Arrighi *et al.*, 2003) or $25 \pm 10\%$ obtained in the presence of GM-CSF/TGFβ/TNFα. In terms of cell numbers, $10^6$ CD34$^+$ progenitors yield on average $30 \times 10^6$ DDL-DC from which we obtain about $10 \times 10^6$ CD1a$^+$ Langerin$^+$ cells. Similar numbers of LC-like DC were reported when culturing CD34$^+$ progenitors directly in GM-CSF/TGFβ/TNFα (Caux *et al.*, 1992). The cells displayed an immature phenotype but responded to DC maturation signal LPS/anti-CD40 by expression of maturation markers CD80, CD83, CD86 as well as CCR7 and HLA-DR. Maturation was accompanied by reduction of Langerin expression, confirming observations made by others (Valladeau *et al.*, 1999; Geissmann *et al.*, 2002).

Addition of IL-4 to GM-CSF/TGFβ antagonized LC differentiation and skewed DC differentiation toward the interstitial type lacking Langerin, supporting previous observations (Caux *et al.*, 1999; Guironnet *et al.*, 2002). Interestingly, in the presence of IL-4, interstitial-like DC expressed low levels of CCR7, which may suffice to direct cell migration to draining lymph nodes (Ohl *et al.*, 2004). Addition of TNFα to GM-CSF/TGFβ also antagonized LC differentiation, which could be correlated with a disposition of these cells to differentiate into macrophages in conjunction with the pre-culture in M-CSF. This is particularly evident at TNFα concentrations $>1$ ng/ml resulting in the formation of plastic-adherent CD11b$^{Hi}$ macrophages (data not shown). In this context, it has been shown that CD34$^+$ progenitor-derived day-6 CD14$^+$CD11b$^+$ cells resist differentiation into cells with LC features upon culture in GM-CSF/TGFβ/TNFα and acquire a more macrophage-like phenotype (Jaksits *et al.*, 1999). The simultaneous presence of IL-4 and TNFα could partially alleviate the inhibitory effects leading to a limited restoration of CD1a$^+$ Langerin$^{Hi}$ cells. However, in spite of different dosage combinations of IL-4 and TNFα, we were never able to completely restore the percentage and level of Langerin expression obtained in the GM-CSF/TGFβ condition. In addition, the Langerin$^+$ cells obtained in GM-CSF/TGFβ/IL-4/TNFα expressed CCR7, which further distinguished them from the cells obtained in GM-CSF/TGFβ.

The dermal microenvironment contains numerous cytokines and cell differentiation signals under both resting and inflamed conditions. The cytokines that we have studied in this work (M-CSF, GM-CSF, TGFβ, TNFα, and IL-4) have all been identified as products of dermal cell types including endothelium, fibroblasts, and mast cells. DDL-DC are model cells for CD14$^+$ DC-SIGN$^+$ cells, which reside in connective tissue such as the dermis under non-inflammatory conditions, where M-CSF is likely to be present. Even under these conditions, LC continue to migrate to draining lymph nodes, and we suggest that dermal CD14$^+$ DC-SIGN$^+$ cells contribute to LC renewal in the steady state.

## MATERIALS AND METHODS

### Cell preparation

Cord blood was obtained from the Bourg-La-Reine maternity ward, France, after informed consent. CD34$^+$ progenitors were immunomagnetically isolated from umbilical cord blood and cultured for 5 days in complete medium composed of RPMI 1640 medium (Gibco-Invitrogen, Cergy-Pontoise, France) with 10% LPS-free fetal calf serum (Gibco-Invitrogen), supplemented with 25 ng/ml stem cell factor (specific activity: $10^6$ U/mg; R&D Systems, Abbington, UK), 3 ng/ml TNFα ($10^8$ U/mg; R&D Systems), and 200 U/ml GM-CSF ($10^7$ U/mg; Schering-Plough, Kenilworth, NJ) (Caux *et al.*, 1996; Cremer *et al.*, 2002). Cells were washed and DDL-DC were obtained after 6 additional days of culture in complete medium supplemented with 25 ng/ml M-CSF ($15 \times 10^7$ U/mg; R&D Systems), with additional M-CSF after 3 days. The CD14$^+$ DDL-DC were immunomagnetically purified using CD14$^+$-coupled magnetic beads (Miltenyi, Bergisch-Gladbach, Germany). DDL-DC were differentiated for 3 days in complete medium with 530 U/ml of GM-CSF (Schering-Plough) and 4 ng/ml of TGFβ ($3 \times 10^7$ U/mg; R&D Systems). Where indicated, 1 ng/ml IL-4 ($24 \times 10^6$ U/mg; Schering-Plough) and/or 0.5–1 ng/ml TNFα (R&D Systems) was added. Cells were matured by incubation with 100 ng/ml LPS and 10 μg/ml anti-CD40 antibody (G24.5) for 24 hours.

### Phenotypic analysis

Expression of specific markers was determined using the following antibodies from Becton-Dickinson-Pharmingen (Le Pont-de-Claix, France) unless otherwise noted: CD80-FITC (RMMP2), CD86-FITC (RMMP1), Langerin-PE (DCGM4) (Coulter-Immunotech, Villepinte, France), HLA DR-FITC (L243), CD83-FITC (HB15e), CD1a-FITC (HI149), CD14-FITC (MφP9), CCR6-biotin (11A9) and mouse IgM anti-CCR7 (2H4) (followed by F[ab']2 biotin-goat anti-mouse IgM from Jackson Immunoresearch, West Grove, PA and streptavidin-APC from Pharmingen), factor XIIIa (Calbiochem, San Diego, CA) (followed by PE-goat anti-rabbit Ig from Jackson Immunoresearch), TRANCE (R&D Systems, directly coupled to Cy3), TRANCE-R (N-20 from Santa Cruz, Santa Cruz, CA, followed by FITC-rabbit anti-goat Ig from Jackson Immunoresearch), DC-SIGN (1B10, a kind gift of Ali Amara, Institut Pasteur, France, followed by PE-goat anti-mouse Ig from Jackson Immunoresearch). Specific binding was measured by flow cytometry on a FACSCalibur (Becton-Dickinson-Pharmingen) and analysis was performed using the CellQuest Pro software (Becton-Dickinson).

### Measurement of intracellular calcium

Cells were incubated in PBS supplemented with 10 mM HEPES, 0.5 mM $MgCl_2$ 1 mM $CaCl_2$, 2 μM Fura-2/AM (Molecular Probes, San Francisco, CA), and 2 μM pluronic acid for 30 minutes at 37°C ($5 \times 10^5$ cells/ml). Fura-2/AM-loaded cells were washed and resuspended in 2 ml of the buffer and transferred to a quartz cuvette for measurements. Stimulation was performed using 100 μM of CCL20 and CCL2 (R&D Systems). Fluorescence was measured using a SAFAS spectrofluorometer (SAFAS, Monaco) in cuvettes thermostatically

controlled at 37°C and continuously stirred. Results are expressed in nM of calcium per $10^6$ cells as a function of the ratio of fluorescence at two excitation wavelengths (340 and 380 nm).

### Chemotactic cell migration

Chemotactic cell migration was assessed using a chemotaxis Boyden microchamber with a standard 5 $\mu$m pore polycarbonate filter (Neuroprobe, Gaithersburg, MD). DDL-DC were incubated in GM-CSF/TGF$\beta$ in the presence or absence of IL-4. After 24 hours, $10^5$ cells were placed in the upper chamber and increasing doses of CCL20 (R&D systems) were added to the lower chamber. After 1 hour at 37°C, cells bound to the separating membrane were washed, fixed in methanol, and stained with eosin and hematoxylin. Membrane-bound cells of duplicates were counted.

### Transmission electron microscopy

For electron microscopy, $10^6$ LC-like DC, generated in GM-CSF/TGF$\beta$, were fixed in cacodylate buffer containing 6% of a 25% glutaraldehyde solution and were further processed as described elsewhere (Hanau *et al.*, 1987). Microscopic analysis was performed using a Zeiss EM109 transmission electron microscope.

## CONFLICT OF INTEREST

The author states no conflict of interest.

## ACKNOWLEDGMENTS

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